

## FLUORESCENT HYBRIDIZATION PROBES WITH REDUCED BACKGROUND

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### Field of the Invention

10 The invention originates from the field of real time PCR. More specifically, the invention is directed to an improved pair of FRET Hybridization probes.

### Description of Related Art

15 In kinetic real-time PCR, the formation of PCR products is monitored in each cycle of the PCR. The amplification is usually measured in thermocyclers which have additional devices for measuring fluorescence signals during the amplification reaction. A typical example of this is the Roche Diagnostics LightCycler (Cat. No. 2 0110468). The amplification products are for example detected by means of fluorescent labelled hybridization probes which only emit fluorescence signals when they are bound to the 20 target nucleic acid or in certain cases also by means of fluorescent dyes that bind to double-stranded DNA. A defined signal threshold is determined for all reactions to be analysed and the number of cycles  $C_p$  required to reach this threshold value is determined for the target nucleic acid as well as for the reference nucleic acids such as the standard or housekeeping gene. The absolute or relative copy numbers of the target molecule can be 25 determined on the basis of the  $C_p$  values obtained for the target nucleic acid and the reference nucleic acid (97/46707; WO 97/46712; WO 97/46714).

30 The FRET real time PCR test format is characterized by two single-stranded hybridization probes which are used simultaneously and are complementary to adjacent sites of the same strand of the amplified target nucleic acid. Both probes are labeled with different fluorescent components. When excited with light of a suitable wavelength, a first component transfers the absorbed energy to the second component according to the principle of fluorescence resonance energy transfer such that a fluorescence emission of the second component can be measured when both hybridization probes bind to adjacent 35 positions of the target molecule to be detected. (Alternatively, fluorescence decrease of the FRET donor component may be monitored.) Among all detection formats known in the art, this "FRET-hybridization probe format" has been proven to be highly sensitive, exact

and reliable. The design of appropriate FRET hybridization probe sequences may sometimes be limited by the special characteristics of the target nucleic acid sequence to be detected.

5 Alternatively, it is also possible to use a fluorescent-labeled primer and only one labeled oligonucleotide probe (Bernard, P. S., et al., Anal Biochem 255 (1998) 101-7).

Another application of FRET hybridization probes is melting curve analysis. In such an assay, the target nucleic acid is amplified first in a typical PCR reaction with suitable amplification primers. The hybridization probes may already be present during the amplification reaction or added subsequently. After completion of the PCR-reaction, the 10 temperature of the sample is constitutively increased, and fluorescence is detected as long as the hybridization probe was bound to the target DNA. At melting temperature, the hybridization probes are released from their target, and the fluorescent signal is decreasing immediately down to the background level. This decrease was monitored with an appropriate fluorescence versus temperature-time plot, a first derivative fluorescence 15 versus temperature plot, an exact temperature value can be determined, at which the fluorescence decrease was observed.

It has been observed however, that depending on the type of fluorescent dye which is used, in most cases there exists a reasonable background fluorescence. This results in a limited dynamic range of the assay, especially, if the fluorescent signals are normalized for 20 background fluorescence by any method known in the art.

It is known from literature that double or multiple labeled Fluorescein donor probes enhance the FRET process such that an increased emission of the FRET acceptor compound is achieved. However, the efficiency of this alternative strongly depends on the spatial orientation of the dyes with respect to the donor probe (Okamura, Y., et al., Nucleic 25 Acids Res 28 (2000) E107). Moreover, multiple fluorescent donor oligonucleotides are difficult to purify and the monomeric donor phosphoramidite building blocks for oligonucleotides are expensive starting materials. In addition, this principle is not universal and is limited to base stable dyes where the phosphoramidite approach is feasible.

30 Thus there is a need for FRET Hybridization probes which provide improved signal intensity and a fairly broad dynamic range of detection, independent from the type of labeling dyes which are used.

### Summary of Invention

This problem is resolved by FRET hybridization probes according to the present invention wherein the FRET donor probe itself is carrying a chemical moiety capable of quenching the fluorescent donor moiety.

5 More precisely, the new invention is directed to a pair of FRET hybridization probes hybridizing adjacently to each other on a target nucleic acid. Said pair of FRET hybridization probes consists of a first oligonucleotide carrying a FRET donor entity and a second oligonucleotide carrying a FRET acceptor entity, wherein the first oligonucleotide is carrying at least one second entity, said second entity being a compound which is  
10 capable of quenching fluorescence emission of said donor fluorescent entity.

Preferably, the nucleotide residue of the first oligonucleotide carrying the donor fluorescence entity is also carrying the second entity with the quenching activity. It is also within the scope of the invention, if one member of said pair of FRET hybridization probes may serve as a primer in a nucleic acid amplification reaction such as  
15 the polymerase chain reaction (PCR).

Moreover, the present invention is directed to a method of using the inventive pair of FRET hybridization probes disclosed above, wherein fluorescence emission of either the donor fluorescent entity or emission of the acceptor fluorescent entity is monitored in Real Time. In a specific embodiment, fluorescence emission of said FRET donor entity is  
20 monitored in a first detector channel and fluorescence emission of said FRET acceptor entity is monitored in a second detector channel, such that the fluorescence emission of said FRET acceptor entity can be normalized by the fluorescence emission of said FRET donor entity

### 25 Brief Description of the Figures

#### Fig. 1

F2/F1-fluorescence versus cycle number plot of the real time PCR experiment disclosed in example 3.

+N/-N: with/without a Nitroindole group at the FRET donor

30 a)  $10^8$  copies, b)  $10^3$  copies of target DNA

#### Fig. 2

1<sup>st</sup> derivative of F2/F1-fluorescence versus temperature plot showing the melting curve analysis of the experiment disclosed in example 4.

.+N/-N: with/without a Nitroindole group at the FRET donor

35 a)  $10^8$  copies, b)  $10^3$  copies of target DNA

**Fig. 3**

F2/F1 fluorescence versus cycle number plot of the real time PCR experiment disclosed in example 5.

The upper curve corresponds to detection with using Fluorescein labeled probe carrying an additional G according to SEQ ID NO: 9, whereas the lower curve corresponds to detection with a Fluorescein labeled probe as known in the art according to SEQ ID NO: 8.

**Detailed description of the invention**

In real time PCR using FRET hybridization probes, fluorescence is monitored as a measure for the amount of amplification product which has been synthesized. For obtaining reliable and quantitative data, it has been proven to be advantageous to perform a normalization of the absolute signal for background fluorescence signalling. In order to achieve this, fluorescence emission of the FRET acceptor is monitored in a first detector channel and fluorescence emission of the FRET donor is monitored in a second detector channel. As a consequence, fluorescence emission of the FRET acceptor can be normalized by means of dividing the obtained value by the fluorescence emission of said FRET donor.

However, dependent on the fluorescent dyes which are actually used for creating a FRET pair, the normalized values are sometimes far from being optimal. This results in a low dynamic range of a respective assay. In this context, the inventors observed that this effect is predominantly due to background signalling of the FRET donor moiety. Due to the general idea of the present invention, this problem may be overcome by an appropriate quenching of the FRET donor signal.

In order to achieve this, FRET donor moieties are quenched by bringing one or more quencher molecules in close vicinity to the FRET donor moiety. According to the invention, this is done in such a way that the oligonucleotide carrying the FRET donor moiety in addition carries a second entity which is capable of quenching the FRET donor moiety itself. Preferably, the residue of the nucleotide residue carrying the donor fluorescence entity is also carrying the second entity with the quenching activity.

In the context of this invention it is also possible to use both, energy transfer quenchers as well as electron transfer quenchers (Dietrich, A., et al., J Biotechnol 82 (2002) 211-31). As a consequence, no chemical consensus structure of quencher moieties which may be used according to the invention, can be defined. The quencher should be dark which means that it does not show any interfering fluorescence during the FRET process. Excluded from the present invention, however, are any quenching effects which may at

least theoretically be due to a quenching effect of multiple FRET donor moieties against each other, since this phenomenon is not universal for all FRET donors.

The inventive method improves the options to design a FRET assay and improves signal dynamics of the FRET detection process, since the interfering emission of the FRET donor is significantly reduced. This is especially important, when the spectrum of the FRET donor emission overlaps with the spectrum of the FRET acceptor emission at least partially.

Surprisingly, despite the presence of a quencher moiety close to FRET donor moiety, the FRET donor is still able to act as a FRET partner. Even more surprisingly, compared to the FRET donor fluorescence, the FRET donor energy transfer activity is less reduced under these conditions for all quenchers tested by the inventors so far. Thus, the introduction of a quencher moiety according to the invention significantly reduces background fluorescence of the FRET donor, but does not affect the efficiency of the FRET process itself.

Suitable quenching compounds are any kind of energy transfer quenchers known in the art. Illustrative but not limiting to the invention is the usage of the following compounds:

- Guanosine/ Diaminopurine
- Nitroindole
- Tryptophane
- Dark quenchers like Dabcyl (Kreuzer, K. A., et al., Clin Chem 47 (2001) 486-90)
- Black hole Quenchers (WO 01/86001)

Any of these or other chemical compounds may be used in order to generate the desired quenching effect. Moreover, even multiple quencher compounds may be used. It is also possible to combine different types of quencher molecules, as long as all quenching compounds are linked either directly or via another quencher compound to the nucleotide residue which carries the FRET donor moiety.

A specific embodiment is based on the observation that in some instances guanosine residues may also have a quenching effect (Seidel et al, J. Phys. Chem. 1996 100,5541-5553). In this embodiment, the introduced quenching compound is a terminal G residue of the hybridization probe carrying the FRET donor, which itself does not form a Watson-Crick base pairing interaction when hybridized to the target nucleic acid to be detected. In this case, the terminal G residue itself is labeled with the fluorescent dye representing the FRET donor moiety.

In a first aspect, the present invention is directed to a pair of FRET hybridization probes consisting of a first oligonucleotide carrying at least a FRET donor entity and a second oligonucleotide carrying at least a FRET acceptor entity. Said first oligonucleotide carrying the donor fluorescent entity is carrying at least one second entity, which is a compound 5 capable of quenching fluorescence emission of said donor fluorescent entity.

The position of the nucleotide residue within the first oligonucleotide may be chosen arbitrarily as long as it results in a quenching effect with regard to emission of the FRET donor entity. Preferably, however, the two residues carrying the FRET donor entity and the quencher entity are in close spatial vicinity to each other. For example, these two 10 residues may be adjacent residues. Most preferably, the same residue is labeled with both the fluorescence donor entity and the quenching entity.

Also preferably, one oligonucleotide carrying the first FRET entity is labeled at its 3' terminal residue and the second oligonucleotide carrying the second FRET entity is labeled at its 5' terminal residue such that when both oligonucleotides are hybridized to the 15 target nucleic acid, the fluorescent labels of both FRET entities are brought in close vicinity to each other due to the fact that said terminal residues are base pairing to adjacent residues in the target nucleic acid or at least to residues which are only separated by one or two further residues. In this context, the oligonucleotide which carries the FRET donor moiety together with the inventive quencher compound may be chosen arbitrarily, since 20 these elements can be introduced on both, either the 5' or the 3' end of an oligonucleotide.

In a second aspect, the present invention is directed to a set of at least 3 oligonucleotides, characterized in that a first oligonucleotide and a second oligonucleotide are capable of acting as a pair of amplification primers for a template dependent nucleic acid amplification reaction. Said first oligonucleotide and said third oligonucleotide are 25 each labeled with at least one corresponding member of a FRET pair consisting of a FRET donor entity and a FRET acceptor entity, wherein the oligonucleotide carrying the FRET donor entity is carrying at least one second entity, said second entity being a compound which is capable of quenching fluorescence emission of said donor fluorescent entity. Again, the two residues of the first oligonucleotide carrying the FRET donor entity and the 30 quencher entity are preferably in close spatial vicinity to each other. For example, these two residues may be adjacent residues. Most preferably, the same residue is labeled with both the fluorescence donor entity and the quenching entity.

It is important that upon hybridization of said third oligonucleotide to the amplified target nucleic acid, the labels of said first oligonucleotide and said third 35 nucleotide are brought in close vicinity to each other such that they are separated by no, one, two or only a few nucleotide residues. This may for example be achieved by a

primer/probe design, characterized in that said first oligonucleotide and a third oligonucleotide are partially complementary to each other. In this regard, the term "partially" need to be understood as comprising embodiments, wherein complementarity is restricted to one, two, three or only a few oligonucleotides.

5 Preferably, the oligonucleotide which serves as a primer and at the same time is labeled in order to be able to participate in the FRET, is preferentially labeled at an internal nucleotide residue, because a 3' terminal labeling would inhibit elongation by a template dependent polymerase and thus result in lack of amplification product. On the other hand, a 5' terminal labeling is not applicable as well, since a label at this position would require  
10 an oligonucleotide probe which would be almost complementary to said first oligonucleotide. This again would lead to problems, during the amplification process because the first oligonucleotide and the oligonucleotide probe could already form hybrids prior to the amplification reaction and result in an increased background fluorescence.

15 Preferably, the third oligonucleotide which is used as hybridization probe is labeled at its 5' terminal residue or at its 3' terminal residue with both the FRET donor moiety and the quencher compound according to the invention. Upon hybridization with the amplified target nucleic acid, the fluorescent labels of both FRET entities are brought into close vicinity to each other such that the two labeled nucleotide residues either form base pairs with each other or at least with adjacent residues.

20 In a third aspect, the present invention is directed to compositions comprising the FRET oligonucleotides disclosed above. More precisely, a composition according to the invention comprises a nucleic acid sample and a pair of hybridization probes characterized in that this pair consists of a first oligonucleotide carrying a FRET donor entity and a second oligonucleotide carrying a FRET acceptor entity, wherein said first oligonucleotide carrying the donor fluorescent entity is carrying at least one second entity, said second entity being a compound which is capable of quenching fluorescence emission of said donor fluorescent entity.

30 Alternatively, the composition according to the invention may comprise a set of at least 3 oligonucleotides, characterized in that a first oligonucleotide and a second oligonucleotide are capable of acting as a pair of amplification primers for a template dependent nucleic acid amplification reaction. The first oligonucleotide and the third oligonucleotide are each labeled with one corresponding member of a FRET pair consisting of a FRET donor entity and a FRET acceptor entity, wherein the oligonucleotide carrying the FRET donor entity is carrying at least one second entity, said second entity being a compound which is capable of quenching fluorescence emission of said donor fluorescent entity. Said first oligonucleotide and said third oligonucleotide may  
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preferentially be partially complementary to each other over a string of one, two, three, or a several few residues.

The present invention is also directed to various methods and applications of using the inventive oligonucleotide pairs and compositions disclosed above.

5 More precisely, the present invention is directed to a method for qualitative or quantitative detection of a nucleic acid sequence in a nucleic acid sample, wherein said nucleic acid sample is being hybridized with a pair of FRET hybridization probes consisting of a first oligonucleotide carrying at least a FRET donor entity and a second oligonucleotide carrying at least a FRET acceptor entity. Said first oligonucleotide carrying 10 the donor fluorescent entity is carrying at least one second entity, which is a compound capable of quenching fluorescence emission of said donor fluorescent entity.

In one embodiment, such a method may be a typical hybridization assay. The hybridization may take place either in solution, or alternatively, either the target nucleic acid or one member of the pair of FRET hybridization probes already by immobilized on a 15 solid support. The solid support itself, for example can be a hybridization membrane, a magnet glass bead, a microarray for immobilizing nucleic acids or any other material known in the art.

In another, highly preferred embodiment, a part of said nucleic acid present in the sample is being subjected to a nucleic acid amplification reaction prior or during the 20 hybridization procedure, for example, a polymerase chain reaction (PCR). As a prerequisite, the target nucleic acid comprises a sequence substantially complementary to the sequence of the used hybridization probes. In other words, the used hybridization probes need to hybridize specifically to the target nucleic acid.

25 Alternatively, in case the target nucleic acid is being amplified, a set of at least 3 different oligonucleotides is used. The set is characterized in that a first oligonucleotide and a second oligonucleotide are capable of acting as a pair of amplification primers for a template dependent nucleic acid amplification reaction. Said first oligonucleotide and said third oligonucleotide are each labeled with at least one corresponding member of a FRET pair consisting of a FRET donor entity and a FRET acceptor entity, wherein the 30 oligonucleotide carrying the FRET donor entity is carrying at least one second entity, said second entity being a compound which is capable of quenching fluorescence emission of said donor fluorescent entity.

In addition, the methods disclosed above allow for a monitoring of the 35 amplification reaction in real time by means of monitoring fluorescence emission of either the donor fluorescent entity or emission of the acceptor fluorescent entity during the

amplification reaction itself. Thus real time monitoring allows the generation of kinetic data and facilitates quantitative analysis.

A low background of the FRET donor entity is especially important in cases wherein fluorescence emission of the FRET acceptor entity is normalized by the fluorescence emission of said FRET acceptor entity. Normalization is usually performed by means of dividing the value monitored for fluorescence emission of the FRET acceptor by the value monitored for the fluorescence emission of said FRET acceptor. Consequently, a low background results in a high normalized score.

In this context, the new invention is also directed to a method for qualitative or quantitative detection of a nucleic acid sequence in a nucleic acid sample, characterized in that fluorescence emission of the FRET donor entity is monitored in a first detector channel and fluorescence emission of said FRET acceptor entity is monitored in a second detector channel, further characterized in that the fluorescence emission of said FRET acceptor entity is normalized by the fluorescence emission of said FRET donor entity.

In a further aspect, the present invention is directed to the usage of the FRET hybridization probes disclosed above for melting curve analysis, wherein monitoring of the dissociation of a complex between a target nucleic acid and a hybridization probe allows for the detection of small sequence variants such as single nucleotide polymorphisms.

More precisely, in one aspect, the invention is directed to a method, wherein first, a ternary hybrid complex between the target nucleic acid and the two hybridization probes is formed. Subsequently, the temperature is increased and the thermal dissociation of the ternary complex is determined by means of monitoring fluorescence in real time. In other words, the new invention is also directed to a method for the determination of the melting profile of a hybrid consisting of a target nucleic acid and a pair of FRET hybridization probes as disclosed above, characterized in that the fluorescence emission is determined as a function of temperature.

Alternatively, it is also possible to determine a melting profile of a hybrid consisting of a target nucleic acid and only one oligonucleotide in cases, wherein the first partner of a FRET pair has been introduced into the amplification product upon usage of an appropriately labeled primer, and the second partner of a FRET pair is being provided by an appropriately labeled oligonucleotide, such that thermal dissociation of a binary hybrid complex between the target nucleic acid and the labeled oligonucleotide functioning as a hybridization probe can be monitored.

Also for performing melting curve analysis, according to the invention it has been proven to be advantageous, if fluorescence emission of the FRET acceptor moiety is monitored in a first detector channel and fluorescence emission of the FRET donor moiety

is monitored in a second detector channel, such that the value obtained for fluorescence emission of said FRET acceptor entity can be normalized by the fluorescence emission of said FRET donor entity.

5 In a last aspect, the present invention is directed to a kit comprising a pair of hybridization probes according to the invention.

Such a kit may comprise a set of FRET hybridization probes according to the invention as disclosed above. In addition, it may also contain oligonucleotides capable of acting as a primer pair for a nucleic acid amplification reaction.

10 Precisely, the kit comprises a pair of FRET hybridization probes consisting of a first oligonucleotide carrying a FRET donor entity and a second oligonucleotide carrying a FRET acceptor entity, wherein said first oligonucleotide carrying the donor fluorescent entity is carrying at least one second entity, said second entity being a compound which is capable of quenching fluorescence emission of said donor fluorescent entity. Preferably, the two residues carrying the FRET donor entity and the quencher entity are in close 15 spatial vicinity to each other. For example, these two residues may be adjacent residues. Most preferably, the same residue is labeled with both the fluorescence donor entity and the quenching entity.

20 Alternatively, the kit comprises a set of at least 3 oligonucleotides, characterized in that a first oligonucleotide and a second oligonucleotide are capable of acting as a pair of amplification primers for a template dependent nucleic acid amplification reaction further characterized in that said first oligonucleotide and said third oligonucleotide are each 25 labeled with one corresponding member of a FRET pair consisting of a FRET donor entity and a FRET acceptor entity, wherein the oligonucleotide carrying the FRET donor entity is carrying at least one second entity, said second entity being a compound which is capable of quenching fluorescence emission of said donor fluorescent entity. Again, the two residues of the first oligonucleotide carrying the FRET donor entity and the quencher entity are preferably in close spatial vicinity to each other. For example, these two residues may be adjacent residues. Most preferably, the same residue is labeled with both the fluorescence donor entity and the quenching entity.

30 In addition, a kit according to the present invention may contain at least one additional component such as a nucleic acid polymerase, deoxynucleoside triphosphates or respective analogues and an appropriate buffer which may be used for a template dependent nucleic acid amplification reaction such as PCR. Furthermore, the kit may also comprise software tools such as compact discs carrying computer programs for 35 quantitative analysis of relative or absolute nucleic acid quantification experiments.

The following examples, references, sequence listing and figures are provided to aid the understanding of the present invention, the true scope of which is set forth in the appended claims. It is understood that modifications can be made in the procedures set forth without departing from the spirit of the invention.

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## EXAMPLES

### Example 1

#### **Preparation of PCR primers and probes**

Primers were synthesized on a 1  $\mu$ mol scale on ABI 394 synthesizer using commercially available standard phosphoramidites ( DMTr ibu G, DMTr bzA; DMTr bz C and DMTr T) and the corresponding CPG support. The chemicals for standard synthesis were obtained from GlenResearch. Removal of the oligonucleotides from the solid support and deprotection was carried out with 33 % NH<sub>3</sub> for 8h at 55 °C. Synthesis was performed in the trityl on modus. Purification was done on a RP 18 Oligo R3 4.6 x 50 mm column from Perseptive Biosystems. Buffer A: 0.1M Triethylammonium acetate in water pH 7.0 /MeCN 95:5 buffer B: MeCN. gradient 3 min 20 % B; 12 min 12- 40 % B flow rate 1 ml/min detection 260 nm. Subsequently, the concentrated oligonucleotide solution was treated for 5 min with 80 % Acetic acid at room temperature in order to remove the 5' DMTr protecting group. Afterwards, oligonucleotides were desalted with a RP 18 column and lyophilized in a Speed Vac.

### Example 2

#### **Preparation of a fluorescently labeled probes and probes carrying a Nitroindole moiety**

Oligonucleotide synthesis was performed in the 1  $\mu$ mol range. Commercially available standard phosphoramidites ( DMTr ibu G, DMTr bzA; DMTr bz C and DMTr T) and chemicals for standard synthesis were obtained from Glen Research. The 5' fluorescein was introduced by using commercially available 5' Fluorescein Phosphoramidite (Glen Research (cat no. 10-1916-90). Nitroindole was introduced during synthesis by using commercially available 5Nitro indole Phosphoramidite (Glen Research cat. No. 10-1044-90) As solid support 3' phosphate CPG ( GlenResearch 20-2900-01) was used. Removal of the oligonucleotides from the solid support and deprotection was carried out with 33 % NH<sub>3</sub> for 8h at 55 °C. The solution was evaporated under vacuum. The remainder was dissolved in double distilled water. The labeled oligonucleotide was purified by reversed phase chromatography using a Oligo R3 4.6 x 50 mm column) Chromatography: buffer A: 0.1M Triethylammoniumacetat in water pH 7.0 buffer B: 0.1 M triethylammonium acetate in water/MeCN 1 : 1. gradient 2 min 0 % B in 45 min to 100 %B ( the gradient was

stopped when a product starts to eluate) The product was eluted at approx. 50 % B. flow rate was 1 ml/min detection at 260 nm. The fractions from the labeled oligonucleotide peak were collected and the solvent was removed by using a vacuum centrifuge. The remainder was dissolved in double distilled water and then evaporated again with vacuum centrifuge. This procedure was repeated three times. The pellet was dissolved in water and lyophilized.

5 For preparation of a 3' LC Red 640 labeled oligonucleotide, synthesis was performed in the 1  $\mu$ mol range. Commercially available standard phosphoramidites (DMTr ibu G, DMTr bzA; DMTr bz C and DMTr T) and chemicals for standard synthesis 10 were obtained from Glen Research. A 3' amino group was introduced by using commercially available 3' aminomodifier CPG (ChemGenes cat no. NSS-0004A02CL) as solid support. Removal of the aminomodified oligonucleotides from the solid support and deprotection was carried out with 33 % NH<sub>3</sub> for 8h at 55 °C. The solution was evaporated 15 under vacuum. The labeling and purification was performed according to the procedure described in the pack insert of the commercially available LC Red 640 NHS ester (Roche Applied Science cat no 2015161).

20 3' Fluorescein labeled oligonucleotides were synthesized and purified according to the pack insert of the commercially available LightCycler Fluorescein CPG (Roche Applied Sience cat no. 3138178). 5' LightCycler Red 640 labeled oligonucleotides were synthesized 25 and purified according to the pack insert of the commercially available LightCycler Red 640 NHS Ester (Roche Applied Sience cat no. 2015161).

### Example 3

Quantitative Real time PCR of Factor V DNA with and without a nitroindole-quenched 25 FRET donor

For amplification of a Factor V DNA fragment, a 20 $\mu$ l Real Time PCR reaction mixture was set up as follows:

10<sup>3</sup> or 10<sup>8</sup> copies of a plasmid containing the Factor V gene  
(Gene Bank Accession No: M\_014335)

30 3 mM MgCl<sub>2</sub>  
500nM each primers according to SEQ ID NO: 1 and 2  
200 nM each FRET hybridization probes according to SEQ ID NO: 3 and 4  
PCR components of LightCycler DNA Master Hybridization Probes Kit (Roche Applied Science, Cat. No. 2158825).

35 The probe according to SEQ ID NO: 3 was 3' terminally labeled with LC-Red-640 (Roche Applied Science) according to example 1. The probe according to SEQ ID NO: 4

was 5' terminally labeled with Fluorescein according to example 1 or Fluorescein and a nitroindole moiety according to example 2.

Amplification was performed in a LightCycler instrument (Roche Applied Science) according to the following thermocycling protocol:

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**Table 1:**

	T[°C]	t[sec]	Ramp-rate[°C/sec]	Acquisition	Cycles
Denaturation	95	30	20.0	none	1
Amplification	95	0	20.0	none	
	55	10	20.0	single	45
	72	10	20.0	none	

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Real time monitoring was performed using the 2<sup>nd</sup> derivative threshold method over 45 cycles by means of dividing the absolute signal values obtained in the FRET acceptor channel through the absolute signal values obtained in the FRET donor channel thus resulting in a normalization of fluorescent signalling e.g. with respect to capillary positioning effects.

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The result is shown in fig. 1. As can be seen in the figure, for both copy numbers of target DNA tested (fig.1a: 10<sup>8</sup> copies, fig. 1b: 10<sup>3</sup> copies), usage of a FRET donor hybridization probe carrying a nitroindole group as a quenching moiety resulted in a significantly increased amplification signal.

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#### Example 4

Melting Curve Analysis of Factor V DNA with and without a Nitroindole-quenched FRET donor

Subsequent to the reaction disclosed in example 2, the samples were subjected to a melting curve analysis according the instructions of the LightCycler manual (Roche Applied Sciences) using the following temperature transition protocol:

Table 2:

	T[°C]	t[sec]	Ramp-rate[°C/sec]	Acquisition	Cycles
5	Meltingcurve	95	0	20.0	none
		45	60	20.0	continous 1
		75	10	0.1	none
	Cooling	40	30	20.0	none 1

Fluorescence monitoring was performed by means of dividing the absolute signal values obtained in channel 2 through the absolute signal values obtained in channel 1 and subsequent calculation of the first derivative.

The result is shown in fig. 2. As can be seen in the figure, for both copy numbers of target DNA tested (fig.2a:  $10^8$  copies, fig. 2b:  $10^3$  copies), usage of a FRET donor hybridization probe carrying a nitroindole group as a quenching moiety resulted in significantly increased melting peaks.

#### Example 5

##### Quantitative Real time PCR of Her4 cDNA with a G quenched FRET donor

For amplification of a Her4 cDNA, two independent 20 $\mu$ l Real Time PCR reaction mixtures were set up basically as disclosed under example 3 with the following modifications:

- Instead of Taq Polymerase, FastStart Taq Polymerase (Roche Applied Sciences Cat. No. 2 158 264) was used.
- cDNA encoding Her4(Gene Bank Accession No: L07868), reverse transcribed from 20ng total RNA from HeLa cells was amplified.
- 500nM each amplification primers according to SEQ ID NO: 5 and 6
- 300nM each FRET hybridization probes according to SEQ ID NO: 7 and 8 or, alternatively,
- 300 nM each FRET hybridization probes according to SEQ ID NO: 7 and 9

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The probe according to SEQ ID NO: 7 was 5' terminally labeled with LC-Red-640 (Roche Applied Science). The probes according to SEQ ID NO: 8 and 9 differ in that the probe according to SEQ ID NO: 8 represents a hybridization probe as known in the art which is exactly complementary to the target nucleic acid, whereas the probe according to

SEQ ID NO: 9 represents a hybridization probe according to the invention carrying a 3' terminal G residue which does not hybridize to the target nucleic acid. Both probes were labeled at their 3' terminal residue with Fluorescein.

5       Amplification was performed basically as disclosed in example 3. The result is shown in fig. 3. The upper curve corresponds to the real time monitoring signal using a pair of FRET hybridization probes according to the invention, wherein the Fluorescein labeled probe was carrying an additional G at its 3' end according to SEQ ID NO: 9. The lower curve corresponds to a real time monitoring signal using a pair of FRET hybridization probes as it is known in the art, characterized in that a Fluorescein labeled probe according to SEQ ID NO: 8 which perfectly matches the target sequence was used. It can be concluded that the increased signal intensity using a FRET donor probe according to SEQ ID NO: 9 is due to the quenching characteristics of the additionally introduced G residue with respect to the Fluorescein compound.

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